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A Novel Porous Nylon Biocarrier for Immobilized Bacteria

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A highly porous nylon biocarrier was developed to support immobilized bacteria in bioreactors used to treat liquid wastes. Porosity analyses and scanning electron microscopy showed microbial colonization of accessible pores typically in the range of 100 to 1,200 μm, with some as large as 3.9 mm. A bench-scale packed-bed reactor achieved a *p*-nitrophenol (PNP) removal rate of 5.95 kg of PNP m^{-3} day^{-1} for wastes containing 1,200 mg of PNP $liter^{-1}$. Complete mixing of the biocarrier bed to remove excess surface biomass was routinely achieved with simple air injection. These porous polymer biocarriers are promising as microbial supports in liquid-waste treatment and bioremediation applications.

Chemical-degrading bacteria immobilized in bioreactors can achieve exceptional performance for the biotreatment of chemical industry wastes (2). Earlier studies have shown that bacteria in fluidized-bed reactors (FBRs) and packed-bed reactors (PBRs) can achieve high rates of chemical removal (1, 5, 6, 9, 13), tolerate harsh conditions such as acidity and thermal shocking (3, 6, 7), survive dormancy (6), tolerate surge loadings (5–8), and produce lower levels of biological solids than conventional waste treatment technologies (5). There is strong interest in the full-scale implementation of these systems to biotreat process wastes or composite effluents from chemical manufacturing sites.

The bioreactive beds in PBRs and FBRs are composed of biocarriers colonized with high concentrations of chemical-degrading microorganisms. These biocarriers may provide surface texture or a porous structure which promotes the attachment and retention of chemical-degrading microorganisms (3, 10, 11, 14–17). Although several of these biocarriers have been well proven as biosupports for immobilized bacteria and some are commercially available, there are some common disadvantages. For example, granular activated carbon and most inorganic biocarriers are colonized well by chemical-degrading bacteria but may experience attrition of 5% or more per year and may produce significant levels of biocarrier-derived suspended solids in bioreactor effluents. Furthermore, the removal of excess microbial biomass from these biocarriers, to prevent hydraulic channeling or loss of interstitial fluid volume, is problematic since their high density or fragility often makes vigorous back-flushing, bed mixing, or mechanical biomass removal difficult. In contrast, sand is affordable and nonfragile but requires a significantly longer time for microbial colonization (slower startup), and microorganisms on sand are more prone to performance failure and slow recovery after physical or chemical upsets than microorganisms on other biocarriers (5). Therefore, the objective of this study was to develop a new biosupport which would achieve chemical biodegradation rates similar to those of conventional biocarriers but would have physical characteristics which enabled efficient removal of excess microbial biomass.

A new process was developed to produce porous nylon biocarriers from melted wet nylon. The prototype R533 porous nylon biocarrier was prepared by this process from nylon 6,6 (Vydyne 21; Monsanto Company, St. Louis, Mo.) with 33% (wt/wt) glass fiber for reinforcement. A blend of 33% (wt/wt) chopped glass fibers (0.32-cm length; Certainteed 93B fiberglass) and 67% (wt/wt) nylon 6,6 was prepared with a twin-shell cone blender. The nylon had a moisture content of 0.3 to 0.5% (wt/wt). The melted blends were compounded in a 3.8-cm-diameter, nonvented, single-screw extruder at a temperature of 285°C with extruder barrel temperatures set on a decreasing profile of 295 to 280°C. The porous extrudate was quenched in a water bath and pelletized. The extrusion process developed in this study can be used to modify the size, shape, and composition of porous nylon biocarriers for both PBR and FBR applications. For example, we have recently produced prototype porous nylon biocarriers containing up to 25% (wt/wt) adsorbants, such as activated carbon, and these prototypes are currently being evaluated in our laboratory.

The prototype porous nylon biocarrier R533 was initially examined to determine its physical appearance, porosity, and movement in a laboratory-scale PBR during both gentle and vigorous aeration. The bench-scale PBR in this study was a 3.5-in. (8.9-cm)-inside-diameter Plexiglas column, as previously described (9). Since the R533 porous nylon biocarrier was only slightly more dense than water, the top 2 to 3 in. (ca. 5 to 8 cm) of the biocarrier bed moved freely in the laboratory-scale PBR during normal aeration (750 cm^3/min). A larger volume of air (2,250 to 3,750 cm^3/min) injected into the bottom of the reactor caused the R533 biocarrier bed to mix completely and vigorously. The pore size distribution for the R533 porous nylon biocarrier was determined by mercury intrusion porosimetry with an Autopore mercury intrusion porosimeter (model 9220-II; Micromeritics, Norcross, Ga.). Mercury porosimetry was useful for characterizing pores ranging from <1 to 390 μm and showing differences between the prototype porous nylon biocarrier, Celite R635 diatomaceous earth beads purchased from Manville Corp. (Denver, Colo.), and activated coconut carbon purchased from Charcoal Filtration Media Co. (Inglewood, Calif.). A plot of incremental pore volume (measured as incremental intrusion of mercury) versus pore diameter for the R533 porous nylon biocarrier and the two conventional biocarriers is shown in Fig. 1. Since most of the pores in granular activated carbon are smaller than 4 μm, they are not accessible to microorganisms (10). The Celite R635 diatomaceous earth biocarrier has a pore size range of 1 to 30 μm with a mean pore size of 20 μm. In contrast, the R533 porous nylon

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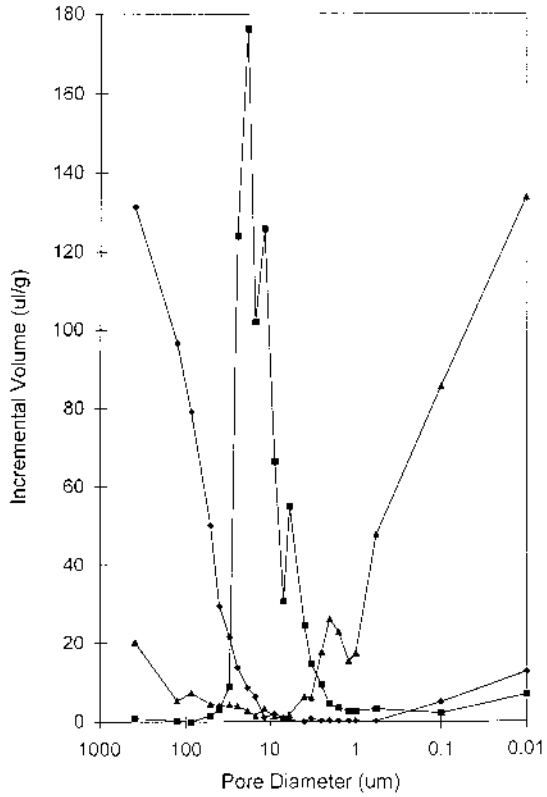

FIG. 1. Porosity of R533 porous nylon biocarrier (◆), Celite R635 diatomaceous earth biocarrier (■), and granular activated carbon (▲) as determined by mercury intrusion porosimetry. Each datum point represents the incremental mercury intrusion for the set of pore volumes ranging in diameter from the plotted value down to the next lower plotted value.

biocarrier had few pores of <1 μm, and the incremental volume of pores was increasing towards 390 μm, the upper limit of the range of pores measured by mercury porosimetry. These results show that the distribution of pores in the porous nylon biocarrier, as measured by mercury porosimetry, was more than an order of magnitude greater than that of either of the common biosupports.

Since mercury porosimetry measures pore sizes up to only 390 μm, the R533 porous nylon biocarrier was analyzed for macropores by scanning electron microscopy (SEM). The dried samples were placed onto aluminum SEM mounts with double-stick tape and coated with Au-Pd by using a Polaron E5100 coating unit for electron beam conductivity (Polaron Inc., Hatfield, Pa.). The SEM analyses were performed and micrographs were obtained with a JEOL 840 scanning electron microscope (JEOL USA, Peabody, Mass.). Samples containing bacteria were rinsed in a buffered solution (0.1 M sodium cacodylate, pH 7.4), fixed in 2% glutaraldehyde, rinsed in sodium cacodylate buffer solution, and fixed in buffered 2% osmium tetroxide for 1 h. The samples were then rinsed in sodium cacodylate buffer solution, dehydrated with an ethanol series, and critical point dried with liquid carbon dioxide.

The longitudinal sectional view at 15-fold magnification (Fig. 2, top) shows that large pores extend throughout the inside of the biosupport running in parallel to the direction of extrusion. Many of these cavities are 800 to 1,200 μm in length, and some openings of up to 3.9 mm in length are present. The cross-sectional view of the cut end of the biosupport at 70-fold magnification (Fig. 2, bottom) shows that many of the long internal cavities running through the inside of the porous biosupport exist as open channels which may be accessed through openings in the ends of the biosupport. These openings are numerous and evenly distributed across the end of the biosupport and have diameters ranging from 40 to 415 μm, providing ready access to inoculated bacteria. Additional SEM analyses showed that these pore openings occur throughout the interior of the biosupport and are readily accessible to microorganisms by openings through both the cut ends and extruded sides of the biosupport.

A large fraction of the total porosity of the porous nylon biocarrier was accessible to microorganisms. The total pore volume and microbe-accessible pore volume (>4 μm) of the R533 porous nylon biocarrier were 0.487 and 0.445 ml/g, respectively. These measurements show that over 91% of the total pore volume was accessible to bacteria. The actual percentage was even higher, since this calculation was based on mercury intrusion porosimetry analyses which were unable to measure the larger pore openings ranging from 400 to 3,900 μm that were observed in the SEM analyses. The large pores extending throughout the porous nylon biocarrier should provide greater diffusion of chemicals, oxygen, and nutrients to

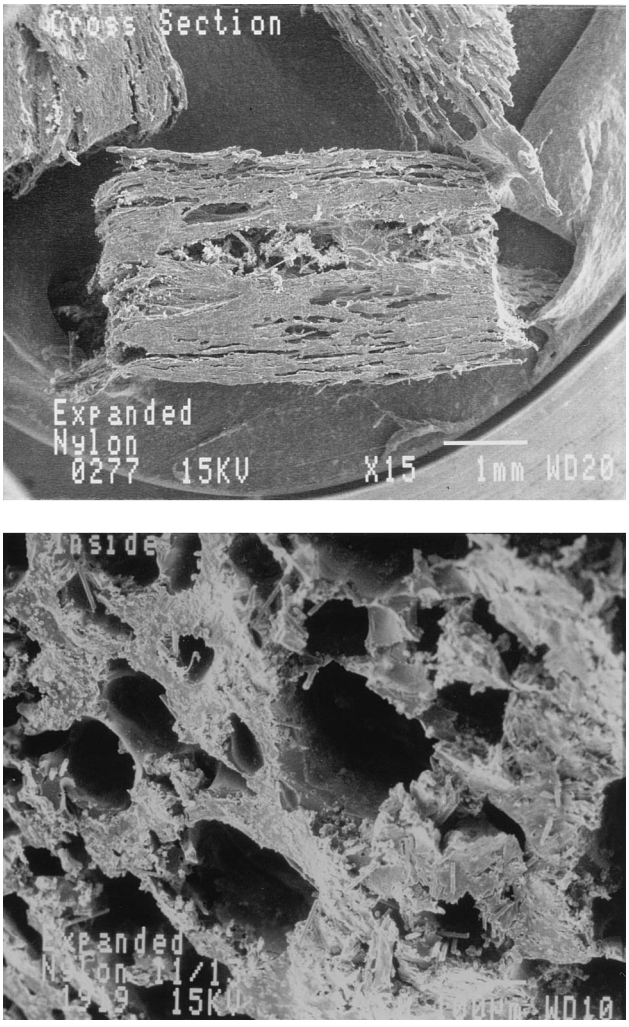

FIG. 2. Scanning electron micrographs of R533 porous nylon biocarrier showing a longitudinal sectional view at ×15 magnification (top) and a cross-sectional cut-end view at ×70 magnification (bottom).

TABLE 1. Comparison of biocarrier density and biodegradation of PNP by immobilized cultures of *P. putida* PNP1 in PBRs

Biocarrier	Biocarrier density (kg/m^3)	PNP loading (kg/m^3/day)	PNP removal (%)	PNP degraded (kg/m^3/day)
R533 porous nylon	323.6	6.60	90.1	5.95
Celite R635 diatomaceous earth	512.6	7.37[a]	91.1[a]	6.71[a]

[a] Data from reference 7.

bacteria inside the biocarrier and more efficient removal of cellular waste products than currently used biocarriers. Repeated SEM analyses showed that microorganisms grew throughout the pores of the nylon biocarriers. In addition, SEM analyses of inoculated biocarriers from a bioreactor operated for over 1 year showed microbial biofilm attached to the internal surface of the open pores throughout the biocarrier but showed no significant occlusion of the internal pore volumes. It is likely that the complete redistribution of biocarrier particles within the PBR by bed mixing provided a sufficient residence time away from the chemical influent point of the plug-flow reactor to allow biofilm degeneration or digestion. This alternating cycle of biomass growth and degeneration would result in porous biocarrier particles, as seen in this study, containing internal pores which were not occluded by biofilm.

Bench-scale PBR studies were conducted to determine whether *Pseudomonas putida* PNP1, a bacterium able to completely degrade *p*-nitrophenol (PNP) as a sole source of carbon and energy (9), would colonize the porous nylon biocarrier and achieve good biodegradation activity. PNP was obtained from Aldrich Chemical Co. (Milwaukee, Wis.), and its purity exceeded 99%. PNP concentrations were determined spectrophotometrically as previously described (7, 9). The porous nylon biocarrier was inoculated by recycled pumping of 1 liter of a moderately turbid 24-h culture of *P. putida* PNP1 in half-strength mineral salts (12) containing 100 mg of PNP per liter at 1 ml/min through the PBR for 24 h at room temperature with continuous aeration. Once microbial degradation of PNP was observed, a metered liquid pump (model RHSY) from FMI Corporation (Oyster Bay, N.Y.) delivered synthetic wastes into the PBR at calibrated flow rates throughout the study. Chemical loading into the PBR was increased by raising the concentration of PNP in the feedstock to approximately 1,200 mg/liter while maintaining a feed rate of 2 ml/min. Continuous aeration maintained oxygen saturation of liquid throughout the PBR during the study period.

A comparison of biocarriers and PNP removal rates achieved by *P. putida* PNP1 in PBRs containing R533 porous nylon biocarrier or Celite R535 diatomaceous earth is summarized in Table 1. Although the PBR containing Celite R635 diatomaceous earth biocarrier achieved 11.5% greater maximum PNP removal than the PBR containing R533 porous nylon biocarrier, the performance of the PBR containing the diatomaceous earth biocarrier dropped to <50% removal of PNP after 3 months because of accumulation of excess biomass in the bed. Analyses of the diatomaceous earth biocarrier by SEM showed a predominance of bacterial growth as a thick surface biofilm, and excess biomass was not removable by air sparging at rates of up to 3,750 cm^3/min through the PBR. The PBR containing the porous nylon biocarrier was completely mixed by air injection at least three times weekly beginning on day 19 to prevent liquid channeling or displacement of interstitial liquid volume due to accumulation of excess surface biomass. This PBR maintained PNP removal rates for over 6 months with no loss of performance due to excess surface biomass and no detectable attrition of biocarrier or occurrence of biocarrier-derived suspended solids in biotreated effluents. SEM analyses showed minimal microbial biomass on the external surface of the R533 porous nylon biocarrier from this PBR.

Biomass levels in the porous nylon biocarrier were estimated by indirect measurement. This approach was required since porous nylon was not amenable to crushing followed by microbial plating or ATP analyses as done with inorganic mineral-based biocarriers (3, 4) or ashing of microbial biomass for gravimetric measurement as dry weight as done with sand or granular activated carbon (5). Measurement of microbial respiration as an indicator of microbial activity showed that R533 porous nylon biocarrier inoculated with *P. putida* PNP1 had resting oxygen uptake rates in this study ranging from 22 to 28 mg of O_2 per liter per h. This was equivalent to typical resting oxygen uptake in a conventional activated-sludge reactor containing 10 g of biomass per liter. This level of biomass was also supported by comparison of PNP biodegradation rates of bacteria immobilized on porous nylon biocarrier and free cell suspensions. Since these levels of microbial colonization and activity were achieved with a porous nylon biocarrier that was frequently mixed and had minimal exterior surface biofilm observable by SEM, these data suggest that chemical removal was achieved primarily by bacteria residing inside the porous nylon biocarrier.

A sample of microorganisms collected from the R533 porous nylon biocarrier after inoculation and at the conclusion of the chemical loading study was diluted and spread plated onto standard plate count agar and mineral salts agar plates containing 100 mg of PNP per liter. Standard plate count agar and purified agar were obtained from Difco Laboratories (Detroit, Mich.). One major bacterial type was isolated from the R533 porous nylon biocarrier at the conclusion of these experiments. This isolate was a gram-negative rod which degraded PNP and was confirmed to be *P. putida* by a Vitek AMS microbial identification system (McDonnell Douglas Inc., St. Louis, Mo.).

The use of fixed-film bioreactors has been shown to be highly effective for the biotreatment of chemical industry wastewaters (2, 5, 6, 8, 9). However, limitations related to the biosupports available for these reactors have been an issue for full-scale implementation and long-term sustained performance. The new porous nylon biocarrier reported in this study supported chemical degradation rates similar to a conventional biocarrier but provided advantages in total porosity, control of excess biomass, and elimination of significant biocarrier losses by attrition. In addition, the porous nylon biocarrier may be produced from nylon manufacturing wastes or from recycled nylon sources, such as used carpets. This new nylon biocarrier should facilitate the advancement of immobilized-bacterium technology.

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REFERENCES

1. **Boucquey, J. B., P. Reynard, P. Amerlynck, P. Modesto Filho, S. N. Agathos, H. Naveau, and E. J. Nyns.** 1995. High-rate continuous biodegradation of concentrated chlorinated aliphatics by a durable enrichment of methano-

genic origin under carrier-dependent conditions. Biotechnol. Bioeng. **47:** 298–307.
2. **Cassidy, M. B., H. Lee, and J. T. Trevors.** 1996. Environmental applications of immobilized microbial cells: a review. J. Ind. Microbiol. **16:**79–101.
3. **Durham, D. R., L. C. Marshall, J. G. Miller, and A. B. Chmurny.** 1994. Characterization of inorganic biocarriers that moderate system upsets during fixed-film biotreatment processes. Appl. Environ. Microbiol. **60:**3329–3335.
4. **Durham, D. R., L. C. Marshall, J. G. Miller, and A. B. Chmurny.** 1994. New composite biocarriers engineered to contain adsorptive and ion-exchange properties to improve immobilized-cell bioreactor process dependability. Appl. Environ. Microbiol. **60:**4178–4181.
5. **Edwards, D. E., W. J. Adams, and M. A. Heitkamp.** 1994. Laboratory-scale evaluation of aerobic fluidized bed reactors for the biotreatment of a synthetic, high-strength chemical industry waste stream. Water Environ. Res. **66:**70–83.
6. **Hallas, L. E., W. J. Adams, and M. A. Heitkamp.** 1992. Glyphosate degradation by immobilized bacteria: field studies with industrial wastewater effluent. Appl. Environ. Microbiol. **58:**1215–1219.
7. **Heitkamp, M. A., W. J. Adams, and V. Camel.** 1993. Evaluation of five biocarriers as supports for immobilized bacteria: comparative performance during high chemical loading, acid shocking, drying and heat shocking. Environ. Toxicol. Chem. **12:**1013–1023.
8. **Heitkamp, M. A., W. J. Adams, and L. E. Hallas.** 1992. Glyphosate degradation by immobilized bacteria: laboratory studies showing feasibility for glyphosate removal from wastewater. Can. J. Microbiol. **38:**921–928.
9. **Heitkamp, M. A., V. Camel, T. J. Reuter, and W. J. Adams.** 1990. Biodegradation of *p*-nitrophenol in an aqueous waste stream by immobilized bacteria. Appl. Environ. Microbiol. **56:**2967–2973.
10. **Kindzierski, W. B., M. R. Gray, P. M. Fedorak, and S. E. Hrudey.** 1992. Activated carbon and synthetic resins as support material for methanogenic phenol-degrading consortia—comparison of surface characteristics and initial colonization. Water Environ. Res. **64:**766–775.
11. **Kuroda, M., M. Yuzawa, Y. Sakakibara, and M. Okamra.** 1988. Methanogenic bacteria adhered to solid supports. Water Res. **22:**653–656.
12. **Leadbetter, E. R., and J. W. Foster.** 1958. Studies on some methane-utilizing bacteria. Arch. Mikrobiol. **30:**91–118.
13. **Nawaz, M. S., W. Franklin, and C. E. Cerniglia.** 1994. Degradation of aliphatic amide mixture by immobilized and nonimmobilized cells of *Pseudomonas sp*. Environ. Sci. Technol. **28:**1106–1109.
14. **Pirbazari, M., T. C. Voice, and W. J. Weber.** 1990. Evaluation of biofilm development on various natural and synthetic media. Hazard. Waste Hazard. Mater. **7:**239–250.
15. **Shimp, R. J., and F. K. Pfaender.** 1982. Effects of surface area and flow rate on marine bacterial growth in activated carbon columns. Appl. Environ. Microbiol. **44:**471–477.
16. **Shreve, G. S., R. H. Olsen, and T. M. Vogel.** 1991. Development of pure culture biofilms of *P. putida* on solid supports. Biotechnol. Bioeng. **37:**512–518.
17. **Wijffels, R. H., and J. Tramper.** 1995. Nitrification by immobilized cells. Enzyme Microb. Technol. **17:**482–492.